

# TLR Activation Excludes Circulating Naive CD8<sup>+</sup> T Cells from Gut-Associated Lymphoid Organs in Mice

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The trafficking of effector T cells is tightly regulated by the expression of site-specific sets of homing molecules. In contrast, naive T cells are generally assumed to express a uniform pattern of homing molecules and to follow a random distribution within the blood and secondary lymphoid organs. In this study, we demonstrate that systemic infection fundamentally modifies the trafficking of circulating naive CD8<sup>+</sup> T cells. We show that on naive CD8<sup>+</sup> T cells, the constitutive expression of the integrin  $\alpha_4\beta_7$  that effects their entry into GALT is downregulated following infection of mice with *Salmonella typhimurium*. We further show that this downregulation is dependent on TLR signaling, and that the TLR-activated naive CD8<sup>+</sup> T cells are blocked from entering GALT. This contrasts strongly with Ag-experienced effector T cells, for which TLR costimulation in the GALT potentially upregulates  $\alpha_4\beta_7$  and enhances trafficking to intestinal tissues. Thus, TLR activation leads to opposite effects on migration of naive and effector CD8<sup>+</sup> T cells. Our data identify a mechanism that excludes noncognate CD8<sup>+</sup> T cells from selected immune compartments during TLR-induced systemic inflammation.

It is generally thought that CD8<sup>+</sup> T cell trafficking to the different parts of the organism is orchestrated at the level of effector T cells (1). During their activation and differentiation following recognition of a specific Ag, effector CD8<sup>+</sup> T cells acquire a site-specific homing phenotype that directs them to different tissue compartments (2). Such channeling of CD8<sup>+</sup> T cells is of particular importance for the gastrointestinal tract and the skin, which form the largest interfaces of the body with the environment. In contrast to the organ-specific migration of effector CD8<sup>+</sup> T cells to peripheral tissues, naive CD8<sup>+</sup> T lymphocytes continuously recirculate to secondary lymphoid organs throughout the organism (1, 3). Under homeostatic conditions the entry of naive T cells into GALT, such as the mesenteric lymph nodes or Peyer's patches, is dependent on the  $\alpha_4\beta_7$  integrin, which is expressed constitutively at low but functionally important levels on naive T cells (4, 5). At the same time, these cells express L-selectin

(CD62L), which is essential for their entry into peripheral lymph nodes (6) but also plays a role in T cell homing to mesenteric lymph nodes and Peyer's patches (3).

In the event of an infection the modifications in the homing patterns of Ag-primed T cells are well described (7, 8), whereas the migratory fate of CD8<sup>+</sup> T cells that do not meet their cognate Ag remains elusive. These naive lymphocytes, which make up most CD8<sup>+</sup> T lymphocytes in secondary lymphoid organs, are exposed to proinflammatory cytokines secreted by activated dendritic cells (DCs) in the inflamed lymphoid tissue milieu (9). Without the necessity of meeting a nominal Ag, naive and memory CD8<sup>+</sup> T cells are stimulated by the induced cytokines in a phenomenon referred to as "bystander" activation (10, 11). Bystander-activated CD8<sup>+</sup> T cells in mice strongly upregulate the phenotypic activation marker CD69 and show increased responsiveness to subsequent stimulation through the TCR (12). These cells also upregulate the chemokine receptor CCR5, which allows them to efficiently find Ag-presenting DCs in the reactive lymph node (13). Whether bystander activation alters the global migratory pattern of naive CD8<sup>+</sup> T cells to different parts of the organism is however not known.

In this study, we show for the first time to our knowledge that TLR activation essentially modifies the homing phenotype of naive bystander-activated CD8<sup>+</sup> T cells. Our data demonstrate that the constitutive expression of the homing integrin  $\alpha_4\beta_7$  is specifically downregulated on non-cognate CD8<sup>+</sup> T cells during infection. We suggest that non-cognate CD8<sup>+</sup> T cells are thus excluded from recirculation to distinct immune compartments such as GALT during acute inflammatory responses.

## Materials and Methods

### Mice

Female C57BL/6 mice were purchased from Harlan-Winkelmann and Janvier. IL-6<sup>-/-</sup> and IL-12p40<sup>-/-</sup> mice were purchased from The Jackson Laboratory. IL-10<sup>-/-</sup> and MyD88<sup>-/-</sup> mice were provided by Dr. J. Heesemann (Munich, Germany). TCR transgenic OT-I mice were provided by Dr. T. Brocker (Munich, Germany). Mice were 8 to 14 wk of age at the onset of experiments. All animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

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Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; CD62L, L-selectin; DC, dendritic cell; MAdCAM, mucosal vascular addressin cell adhesion molecule.

## Flow cytometry

Abs to CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), CD11c (N418), CD19 (6D5), CD44 (IM7), CD69 (H1.2F3),  $\alpha_4\beta_7$  (DATK32), CD62L (MEL-14), LFA-1 (H155-78), and CCR7 (4B12) were purchased from BioLegend. The Ab to CCR9 (242503) was purchased from R&D Systems. Cells were stained in PBS supplemented with 10% FCS and were analyzed using a FACSCanto II or FACSCalibur (both BD Biosciences). CD8<sup>+</sup> T cells were characterized by a CD3<sup>+</sup>CD19<sup>-</sup>CD8<sup>+</sup>CD4<sup>-</sup> phenotype (Supplemental Fig. 2A). All data were evaluated with FlowJo software (Tree Star).

## Infection assays

For oral infections, the *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) strain SB300 was cultured in 0.3 M NaCl Luria-Bertani medium to allow for the activation of the *Salmonella* type III secretion system. One day prior to infection, mice were orally treated with 20 mg streptomycin to ensure consistent infection conditions. Water and food were withdrawn 4 h before groups of mice were orally immunized with 10<sup>3</sup> CFU (or 10<sup>5</sup> CFU for in vivo migration assays with adoptive T cell transfer) of *S. typhimurium* in PBS by using round-bottom gavage needles. Thereafter, drinking water was offered immediately and food was provided 2 h after immunization. For in vitro infection assays, the *S. typhimurium* strain SB824 was used. Splenocytes were cocultured with either live or heat-inactivated (30 min at 65°C) *S. typhimurium* at a multiplicity of infection of 10 for 2 h in RPMI 1640 containing 1% L-glutamine. For some experiments further multiplicities of infection of 1 and 0.1 were used. After 2 h coculture, medium was exchanged for DMEM medium containing 20% FCS, 1% L-glutamine, and 0.1% gentamicin. In some experiments, splenocytes were stimulated with PMA (10 ng/ml) and ionomycin (1  $\mu$ g/ml; both InvivoGen).

## In vivo TLR stimulation

Mice were injected s.c. either with 100  $\mu$ g fully phosphorothioated CpG oligodeoxynucleotide 1826 (5'-TCCATGACGTTTCCTGACGTT-3'; Coley Pharmaceutical or Eurofins), 50  $\mu$ g LPS from *Escherichia coli*, or 20  $\mu$ g R848 (both InvivoGen). Treated mice were killed 24 h later for analysis unless stated otherwise. In some experiments, mice were treated with repeated injections of 100  $\mu$ g CpG s.c. 7, 5, 3, and 1 d before the absolute CD8<sup>+</sup> T cell count was determined in secondary lymphoid organs by flow cytometry.

## Cell preparation, purification, and in vitro stimulation

Splenocytes and bone marrow-derived DC (BMDC) single-cell suspensions were prepared as described previously (14). Splenic CD8<sup>+</sup> T cells were negatively enriched using a MACS CD8<sup>+</sup> T cell isolation kit and LS columns (Miltenyi Biotec) according to the manufacturer's protocol. Splenic DCs were depleted from total splenocytes using CD11c beads (Miltenyi Biotec). Purity of magnetically sorted T cells was >95%. After DC depletion, residual DCs made up <0.05% of total splenic cells. Cells (1  $\times$  10<sup>6</sup> cell/ml) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100  $\mu$ g/ml streptomycin, and 1 IU/ml penicillin (complete RPMI 1640) for 24–36 h with LPS (1  $\mu$ g/ml), Pam<sub>3</sub>CSK4 (3  $\mu$ g/ml), flagellin (2  $\mu$ g/ml), R848 (5  $\mu$ g/ml; all InvivoGen), or CpG (3  $\mu$ g/ml). For coculture experiments of purified CD8<sup>+</sup> T cells and BMDCs, 0.4- $\mu$ m pore size transwell culture inserts (Nunc) were used to prevent direct cell–cell contact. For some experiments, splenocytes were stimulated for 24 h with recombinant IL-6, IL-10, or IL-12p70 (all PeproTech) at the indicated concentrations.

## Quantification of cytokines

Concentrations of IL-4, IL-6, IL-10, and IL-12p70 in splenocyte culture supernatants were determined by ELISA according to the manufacturer's instructions (BD Biosciences). The protocol for IFN- $\alpha$  measurement by sandwich-ELISA was described previously (15). In brief, rat mAb against mouse IFN- $\alpha$  (clone RMMA-1) was used as the capture Ab, rabbit polyclonal Ab against mouse IFN- $\alpha$  was used for detection (both from PBL Biomedical Laboratories) together with HRP-conjugated donkey Ab to rabbit IgG as the secondary detection reagent (Jackson ImmunoResearch). Recombinant mouse IFN- $\alpha$  (PBL Biomedical Laboratories) was used as standard.

## Mucosal vascular addressin cell adhesion molecule-1 adhesion assay

Ninety-six-well plates were coated with recombinant mouse mucosal vascular addressin cell adhesion molecule (MAdCAM)-Fc chimera (10  $\mu$ g/ml;

R&D Systems) overnight at 4°C. The supernatant was then discarded and wells were blocked with 1% BSA in PBS for 30 min at room temperature. Mice were injected with 100  $\mu$ g CpG s.c. and 24 later CD8<sup>+</sup> T cells were purified from freshly isolated splenocytes. T cells and TK-1 cells were labeled with CFSE according to the manufacturer's protocol. Cells (10<sup>7</sup>/ml in DMEM without phenol supplemented with 25 mM HEPES) were preincubated in polypropylene tubes with or without DATK32 Ab (10  $\mu$ g/ml) for 15 min at 37°C before cells were added to coated plates and allowed to settle for 25 min at 37°C and 5% CO<sub>2</sub>. Nonadherent cells were removed by two to three washing steps with PBS. Fluorescent emission of adhesive cells was measured with a plate reader.

## Short-term in vivo homing of CD8<sup>+</sup> T cells

Splenocytes were cultured in complete RPMI 1640 with or without added CpG (3  $\mu$ g/ml) for 36 h. Subsequently, the two cell cultures were stained with different CFSE intensities (1 and 10  $\mu$ M CFSE staining concentration). Cells (1–2  $\times$  10<sup>7</sup>) from each preparation (CpG-activated CFSE<sup>high</sup> and unstimulated CFSE<sup>low</sup> splenocytes) were mixed and injected i.v. into naive recipient mice. An aliquot was saved to assess the input ratio. Eight hours after the adoptive transfer, cell preparations from different recipient tissues were analyzed for adoptively transferred CD8<sup>+</sup> T cells by flow cytometry. The homing index was calculated as the ratio of [CFSE<sup>high</sup>]<sub>tissue</sub>/[CFSE<sup>low</sup>]<sub>tissue</sub> to [CFSE<sup>high</sup>]<sub>input</sub>/[CFSE<sup>low</sup>]<sub>input</sub>. (16) For in vivo homing analysis during *S. typhimurium* infection, freshly isolated splenocytes were labeled with CFSE and adoptively transferred into recipient mice 1 d prior to bacterial inoculation. Two days after infection mice were sacrificed and numbers of transferred CD8<sup>+</sup> T cells in secondary lymphoid organs were analyzed. For the determination of CD8<sup>+</sup> T cell numbers in secondary lymphoid organs, bilateral inguinal and axillary lymph nodes were pooled (peripheral lymph nodes). For mesenteric lymph nodes, all lymph nodes along the full length of the superior mesenteric artery to the aortic root were dissected as described (17). All Peyer's patches were prepared and were pooled for further analysis.

## OVA immunization and in vivo proliferation

Magnetically purified splenic CD8<sup>+</sup> T cells from wild-type or OT-I transgenic mice were labeled with CFSE or the CellTracker Violet (both 5  $\mu$ M; Molecular Probes) according to the manufacturer's protocol and mixed in equal numbers. Naive wild-type mice were injected i.v. with  $\sim$ 1  $\times$  10<sup>7</sup> total CD8<sup>+</sup> T cells. Twenty-four hours after adoptive transfer, recipient mice were immunized i.p. with 100  $\mu$ g CpG or LPS and 500  $\mu$ g OVA. Thirty-eight hours later,  $\alpha_4\beta_7$  expression on adoptively transferred CD8<sup>+</sup> T cells was assessed in secondary lymphoid organs by flow cytometry. Owing to fluorescent dye restrictions, in some experiments CD8<sup>+</sup> T cells from wild-type and OT-I mice were labeled with eFluor 670 (Molecular Probes) and were separately transferred into naive mice. Recipient mice were immunized with CpG and OVA and cell proliferation was analyzed 42 h later by flow cytometry.

## Statistical analysis

All data are presented as means  $\pm$  SEM. Statistical significance of single experimental findings was assessed with the independent two-tailed Student *t* test. For multiple statistical comparison of a data set, the one-way ANOVA test with Bonferroni posttest was used. A *p* value <0.05 was considered to be statistically significant. All statistical calculations were performed using Graphpad Prism (GraphPad Software).

## Results

### *S. typhimurium* infection downregulates $\alpha_4\beta_7$ on CD8<sup>+</sup> T cells

To investigate whether bacterial stimulation alters the migration potential of naive CD8<sup>+</sup> T lymphocytes, we examined the expression of lymphocyte homing molecules following coculture of murine splenocytes with *S. typhimurium*. CD62L is of critical importance to naive T cells to enter skin-draining peripheral lymph nodes but it also plays a role in their migration to GALT (6). The chemokine receptor CCR7 and the LFA-1 are homing molecules that support naive T cell trafficking to both intestinal and non-intestinal lymphoid tissues (18). Consistent with previous reports, flow cytometry analysis showed that naive CD8<sup>+</sup> T cells uniformly express  $\alpha_4\beta_7$  at relatively low levels (Fig. 1A) as well as CD62L, CCR7, and LFA-1. Following coculture of naive splenocytes with *S. typhimurium*, the gut-homing integrin  $\alpha_4\beta_7$  was significantly

downregulated on CD8<sup>+</sup> T cells (Fig. 1A). In contrast, the other homing molecules examined were not altered. To investigate whether the downregulation of  $\alpha_4\beta_7$  occurs in the context of systemic infection, we next examined the expression of  $\alpha_4\beta_7$  by CD8<sup>+</sup> T cells in secondary lymphoid organs after oral infection of mice with *S. typhimurium*. This model mimics typhoid-like disease and is characterized by rapid systemic spread and multiplication of bacteria in the liver and spleen. We observed a consistent loss of surface  $\alpha_4\beta_7$  expression in the spleen and peripheral lymph nodes as well as in the GALT 24 h after infection (Fig. 1B). Thus, following *S. typhimurium* infection both in vitro and in vivo, CD8<sup>+</sup> T cells selectively downregulate  $\alpha_4\beta_7$  among the molecules that effect their migration to secondary lymphoid organs.

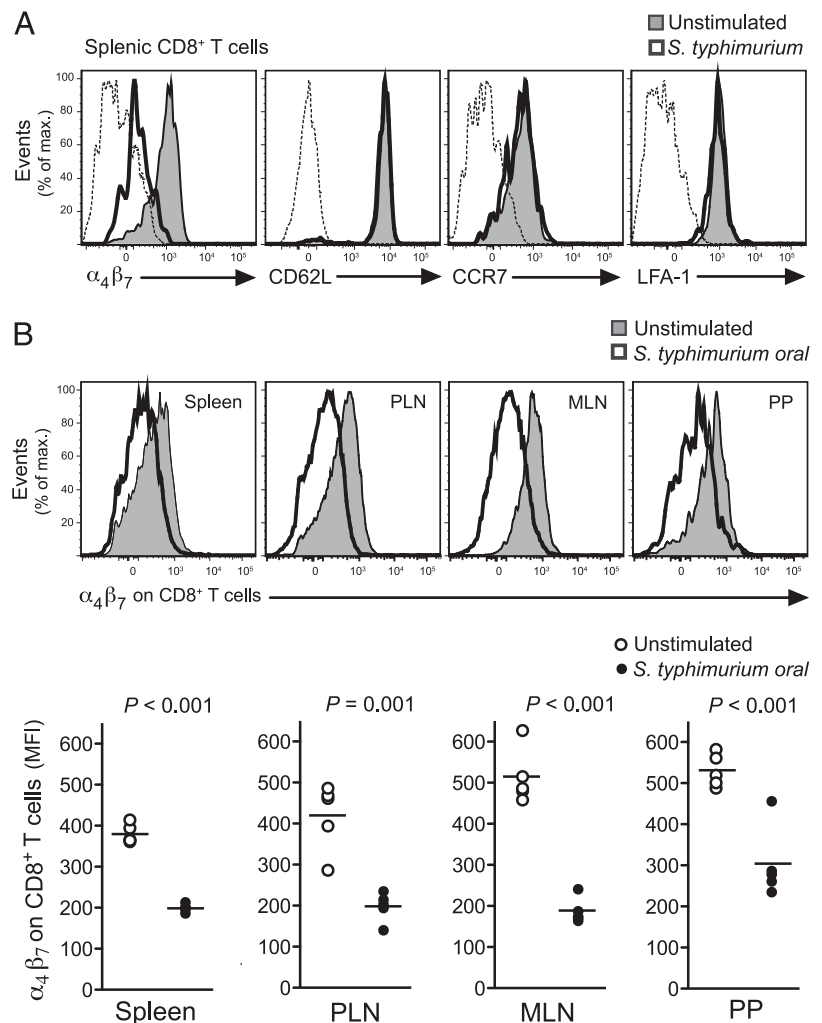
#### The downregulation of $\alpha_4\beta_7$ following *S. typhimurium* infection is due to TLR activation

To investigate the molecular mechanism by which *S. typhimurium* infection blocks the expression of  $\alpha_4\beta_7$ , we inoculated splenocyte cultures with *S. typhimurium*. We observed that not only live but also heat-inactivated bacteria impaired the expression of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells, indicating that downregulation of  $\alpha_4\beta_7$  by *S. typhimurium* is independent of bacterial virulence factor expression, replication, or metabolism (Supplemental Fig. 1A, 1B). Pattern recognition receptors, in particular TLRs, are critically involved in sensing *S. typhimurium* and in the initiation of the subsequent immune response (19, 20). To examine whether TLRs play a role in the regulation of  $\alpha_4\beta_7$  during bacterial infection, we cocultured

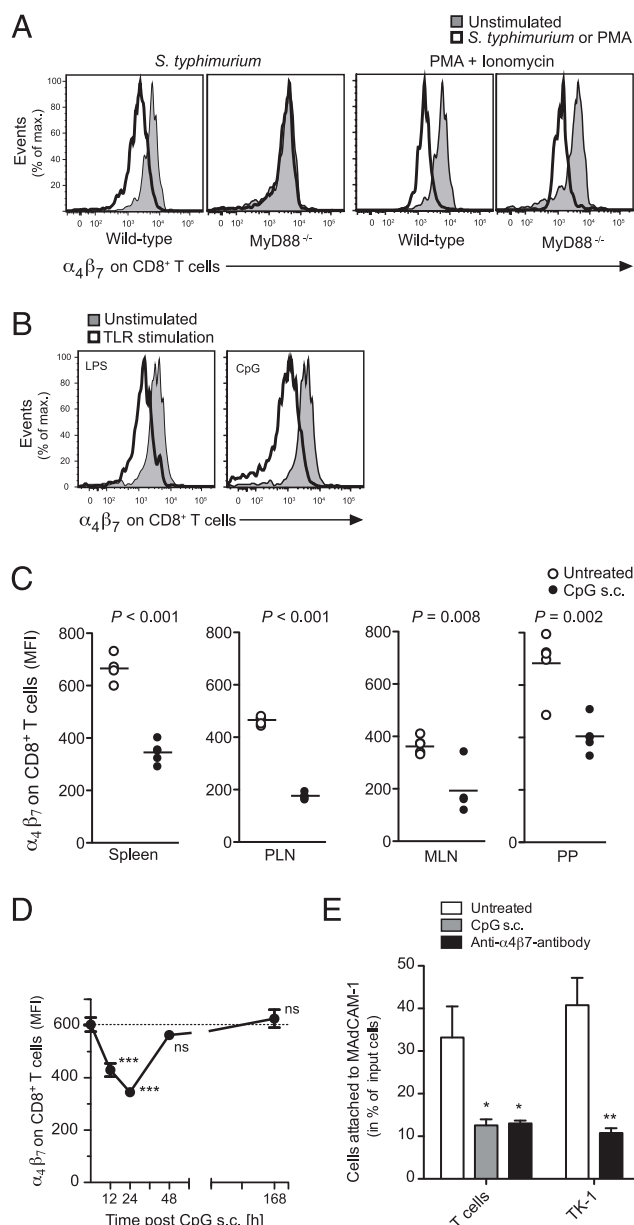
splenocytes from wild-type and MyD88-deficient mice with *S. typhimurium*. MyD88 is a central adaptor protein that is crucial for the downstream signaling of most TLRs, which culminates in the activation of the proinflammatory transcription factor NF- $\kappa$ B (21). Upon bacterial infection, we observed no change in surface  $\alpha_4\beta_7$  levels on CD8<sup>+</sup> T cells from mice that lack functional MyD88, indicating that  $\alpha_4\beta_7$  downregulation is mediated by TLR signaling (Fig. 2A). In contrast, treatment of splenocytes with PMA, a MyD88-independent activator of protein kinase C and hence of NF- $\kappa$ B, reduced expression of  $\alpha_4\beta_7$  on both wild-type and MyD88-deficient CD8<sup>+</sup> T cells.

To further examine the involvement of TLR signaling in the regulation of  $\alpha_4\beta_7$  expression, we stimulated splenocyte cultures with agonists for TLR2, TLR4, TLR5, or TLR9, as these receptors are known to be activated upon *S. typhimurium* infection (22). The synthetic triacylated lipoprotein Pam<sub>3</sub>CSK4 (TLR1 and TLR2), LPS (TLR4), and the oligodeoxynucleotide CpG DNA (TLR9) all caused a loss of  $\alpha_4\beta_7$  expression that was entirely dependent on MyD88 (Fig. 2B, Supplemental Fig. 1C). In accordance with the fact that murine splenocytes do not express functional TLR5 (23), the TLR5 ligand flagellin did not induce  $\alpha_4\beta_7$  downregulation. We furthermore examined the effect of a TLR7 agonist on  $\alpha_4\beta_7$  expression, as a recent report demonstrated that phagosomal bacteria can induce activation of TLR7 (24). We found that the imidazoquinoline compound R848, a synthetic ligand of TLR7, also downregulated  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells (Supplemental Fig. 1C).

**FIGURE 1.** *S. typhimurium* infection downregulates  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells. **(A)** Flow cytometry analysis of homing molecules expressed by CD8<sup>+</sup> T cells after total splenocytes from naive mice were cultured in the presence of *S. typhimurium* for 36 h (isotype controls, dashed line). **(B)** Expression of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells from mice that were orally infected with *S. typhimurium* 24 h prior to the analysis. Representative histograms are gated on CD8<sup>+</sup> T cells from secondary lymphoid organs. Diagrams show  $\alpha_4\beta_7$  mean fluorescence intensity (MFI) on CD8<sup>+</sup> T cells of individual mice in secondary lymphoid organs. Horizontal bars represent the mean of individual mice ( $n = 5$ ). Results are representative of at least two independent experiments. MLN, Mesenteric lymph node; PLN, peripheral lymph node; PP, Peyer's patches.







**FIGURE 2.** The downregulation of  $\alpha_4\beta_7$  following *S. typhimurium* infection is due to TLR activation. **(A)** Expression of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells after coculture of total splenocytes from naive wild-type and MyD88-deficient (MyD88<sup>-/-</sup>) mice with *S. typhimurium* or stimulation with PMA and ionomycin or **(B)** different TLR ligands. **(C)** Mice were injected once s.c. with the TLR9 ligand CpG. Expression of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells in secondary lymphoid organs was analyzed 24 h later. Diagrams show  $\alpha_4\beta_7$  mean fluorescence intensity (MFI) on CD8<sup>+</sup> T cells of individual mice in secondary lymphoid organs. Horizontal bars represent the mean of individual mice ( $n = 5$ ). **(D)** Expression of  $\alpha_4\beta_7$  on splenic CD8<sup>+</sup> T cells from CpG-injected mice at various time points after treatment. Data points give mean values of individual mice ( $n = 5$ )  $\pm$  SEM. An asterisk indicates comparison with CD8<sup>+</sup> T cells from unstimulated mice. **(E)** Adhesion of freshly isolated splenic CD8<sup>+</sup> T cells from CpG-injected mice or cultured TK-1 cells to plate-bound recombinant MAdCAM-Fc chimera. Splenocytes were incubated with anti- $\alpha_4\beta_7$  Ab as positive control. Data give the mean value of triplicate samples  $\pm$  SEM. Results are representative of at least four (B, D) or two (A, C, E) independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

To examine whether TLR stimulation can also influence  $\alpha_4\beta_7$  expression in vivo, we treated mice with a single injection of CpG and examined  $\alpha_4\beta_7$  expression after the treatment. Indeed, as seen following *S. typhimurium* infection,  $\alpha_4\beta_7$  was decreased on CD8<sup>+</sup>

T cells in all secondary lymphoid organs examined (Fig. 2C). The loss of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells upon TLR activation was reversible, as expression was lowest 24 h after CpG injection and returned to baseline levels at 48 h after application (Fig. 2D). These data collectively suggest that  $\alpha_4\beta_7$  downregulation on CD8<sup>+</sup> T cells following *S. typhimurium* infection is a broad reaction pattern that occurs upon activation of different TLRs and may thus not be specific for this pathogen. Indeed, culture of murine splenocytes in the presence of *Escherichia coli* resulted in a similar downregulation of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells (Supplemental Fig. 1B). To better understand the underlying molecular mechanisms, we used the well-characterized TLR ligands LPS and CpG to further investigate the TLR-induced downregulation of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells.

The ligand for  $\alpha_4\beta_7$ , MAdCAM-1, is expressed by specialized endothelial cells in GALT, thus enabling naive CD8<sup>+</sup> T cells to home to the intestinal immune compartment (2). To test whether TLR-induced  $\alpha_4\beta_7$  downregulation on CD8<sup>+</sup> T cells translates into reduced affinity to MAdCAM-1, the binding capacity of CD8<sup>+</sup> T cells from CpG-treated mice to plate-bound mouse MAdCAM-Fc chimera was determined as described (25). Indeed, freshly isolated splenic CD8<sup>+</sup> T cells from CpG-treated mice showed reduced adhesion to MAdCAM-1, similar to CD8<sup>+</sup> T cells that were treated in vitro with the  $\alpha_4\beta_7$ -blocking Ab DATK32 (Fig. 2E) or CpG (Supplemental Fig. 1D). The  $\alpha_4\beta_7$ <sup>+</sup> mouse TK1 lymphoma cell line was used as a positive control (25).

#### *$\alpha_4\beta_7$ is selectively downregulated on naive bystander-activated CD8<sup>+</sup> T cells*

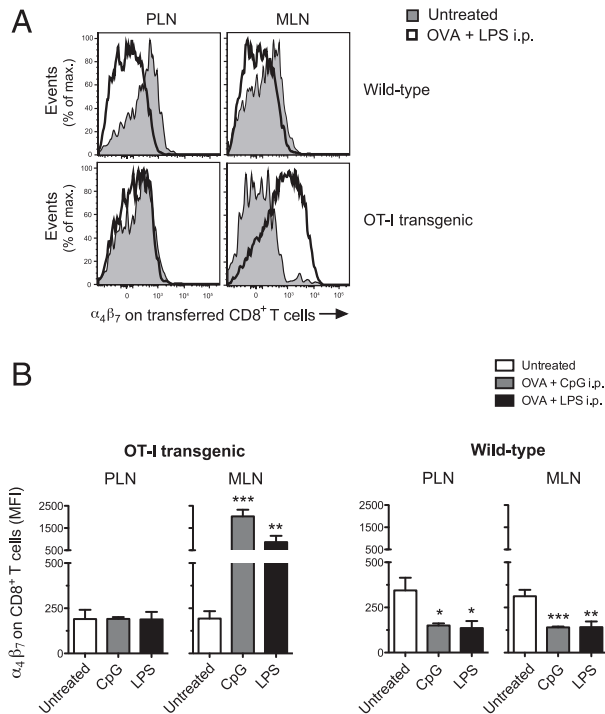
Previous in vitro studies have shown that the ability to home to the gut, characterized by high  $\alpha_4\beta_7$  expression, is most potently imprinted on CD8<sup>+</sup> T cells by DCs from GALT during stimulation through the TCR (16, 26). Following in vivo immunization, GALT DCs only have this capacity in the presence of maturational stimuli, including TLR-dependent and -independent adjuvants (27). In contrast, our data now show that most CD8<sup>+</sup> T cells in GALT and other secondary lymphoid organs actually downregulate the expression of  $\alpha_4\beta_7$  upon stimulation with TLR ligands. Further characterization of the CD8<sup>+</sup> T cell phenotype showed that the high proportion of phenotypically naive (CD44<sup>low</sup>CD62L<sup>high</sup>) cells in secondary lymphoid organs remained unchanged by TLR treatment (Supplemental Fig. 2A). These naive CD8<sup>+</sup> T cells showed downregulation of  $\alpha_4\beta_7$  and simultaneously upregulated the activation marker CD69 (Supplemental Fig. 2C, 2D), resembling a phenotype that is compatible with bystander activation (11). Expression levels of  $\alpha_4\beta_7$  on bystander-activated CD8<sup>+</sup> T cells did not significantly differ when comparing total and naive CD8<sup>+</sup> T cells in all secondary lymphoid organs examined, probably because of the high frequency of naive T cells in these tissues (Supplemental Fig. 2B, 2C). We hypothesized that the expression of the gut-homing receptor  $\alpha_4\beta_7$  in response to these stimuli is differentially regulated on Ag-specifically-activated versus bystander-activated naive T cells.

To investigate the specificity of TLR-induced  $\alpha_4\beta_7$  downregulation, OVA-specific (OT-I) and wild-type splenic CD8<sup>+</sup> T cells were fluorescently labeled with CFSE or CellTracker Violet, mixed, and coinjected i.v. into naive wild-type recipient mice. Eighteen hours after adoptive transfer, mice were immunized with endotoxin-free OVA and either LPS or CpG as adjuvant based on the published models involving Ag-specific T cells (27). Two days after immunization,  $\alpha_4\beta_7$  expression on transferred CD8<sup>+</sup> T cells was analyzed in secondary lymphoid organs. In line with our previous findings, wild-type CD8<sup>+</sup> T cells showed uniform downregulation of  $\alpha_4\beta_7$  in mice immunized with LPS or CpG and OVA (Fig. 3). Transferred wild-type CD8<sup>+</sup> T cells did not proliferate and showed

stable expression of CD62L following immunization with CpG and OVA, confirming their naive phenotype (Supplemental Fig. 2E). In contrast, in the same mice  $\alpha_4\beta_7$  expression on OT-I CD8<sup>+</sup> T cells in mesenteric lymph nodes was strongly upregulated, as demonstrated previously (27) (Fig. 3).  $\alpha_4\beta_7$  upregulation was associated with high proliferation of the OT-I CD8<sup>+</sup> T cells and their coincident loss of CD62L expression, as expected for Ag-specific-activated T cells (Supplemental Fig. 2E). Thus, whereas Ag-specific-activated CD8<sup>+</sup> T cells show site-specific upregulation of  $\alpha_4\beta_7$ , TLR-activated CD8<sup>+</sup> T cells respond with homogeneous downregulation of this integrin.

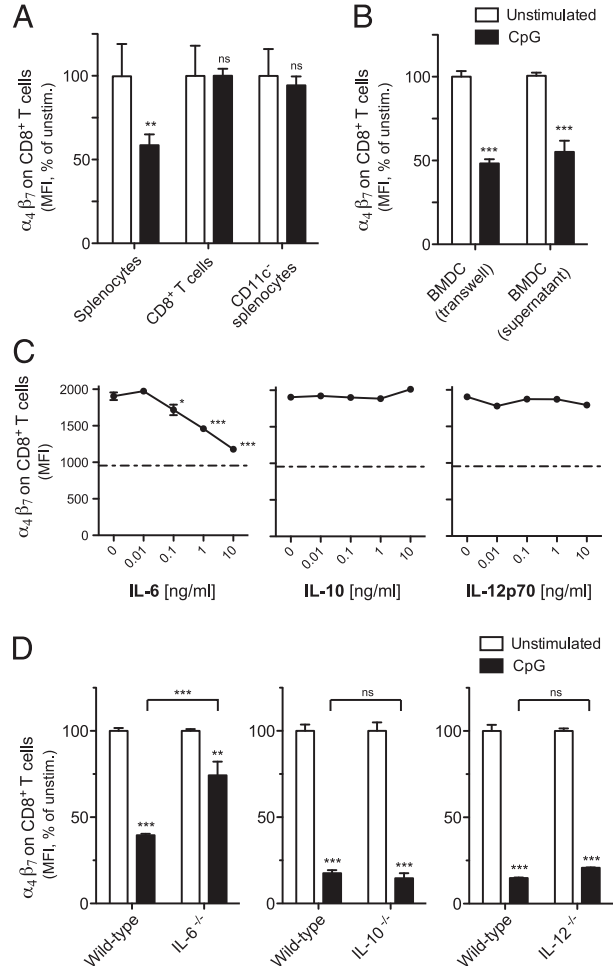
#### $\alpha_4\beta_7$ downregulation is mediated by DCs and is dependent on IL-6

Because of their limited TLR expression (28), CD8<sup>+</sup> T cells do not respond directly to most TLR stimuli (11, 29, 30). Indeed, we demonstrated that purified splenic CD8<sup>+</sup> T cells do not downregulate  $\alpha_4\beta_7$  following stimulation with CpG (Fig. 4A). DCs, in contrast, express a large repertoire of pattern recognition receptors and are thus crucial in linking the innate recognition of pathogens with the initiation of an adaptive immune response. Because DCs are the main cell type that induces bystander activation of CD8<sup>+</sup> T cells following TLR stimulation (11), we investigated whether DCs play a role in  $\alpha_4\beta_7$  downregulation on these cells. Depletion of CD11c<sup>+</sup> DCs abolished downregulation of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells in CpG-activated splenocytes, demonstrating that DCs are



**FIGURE 3.**  $\alpha_4\beta_7$  is selectively downregulated on bystander-activated CD8<sup>+</sup> T cells. Purified splenic CD8<sup>+</sup> T cells from wild-type and OT-I transgenic mice were fluorescently labeled, mixed, and adoptively transferred into wild-type mice. Recipient mice were immunized with LPS or CpG and OVA. Expression of  $\alpha_4\beta_7$  on transferred CD8<sup>+</sup> T cells in peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) was analyzed by flow cytometry 48 h after immunization. (A) Representative histograms are gated on transferred CD8<sup>+</sup> T cells and show the overlay of untreated and immunized mice. (B) Diagrams show  $\alpha_4\beta_7$  mean fluorescence intensity (MFI) of transferred CD8<sup>+</sup> T cells in immunized mice. Data show the mean values of individual mice ( $n = 3$ )  $\pm$  SEM. Results are representative of at least two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

essential for the CpG-induced  $\alpha_4\beta_7$  decrease in vitro (Fig. 4A). Furthermore, coculture of CpG-activated BMDCs and purified CD8<sup>+</sup> T cells in a transwell culture system resulted in downregulation of  $\alpha_4\beta_7$  on T cells (Fig. 4B), demonstrating that direct cell-cell contact is not necessary for the effect of CpG. Addition of culture supernatant from CpG-activated DCs to purified CD8<sup>+</sup> T cells similarly reduced expression levels of  $\alpha_4\beta_7$ , confirming that DC-secreted soluble factors can mediate the downregulation of  $\alpha_4\beta_7$  in vitro (Fig. 4B).



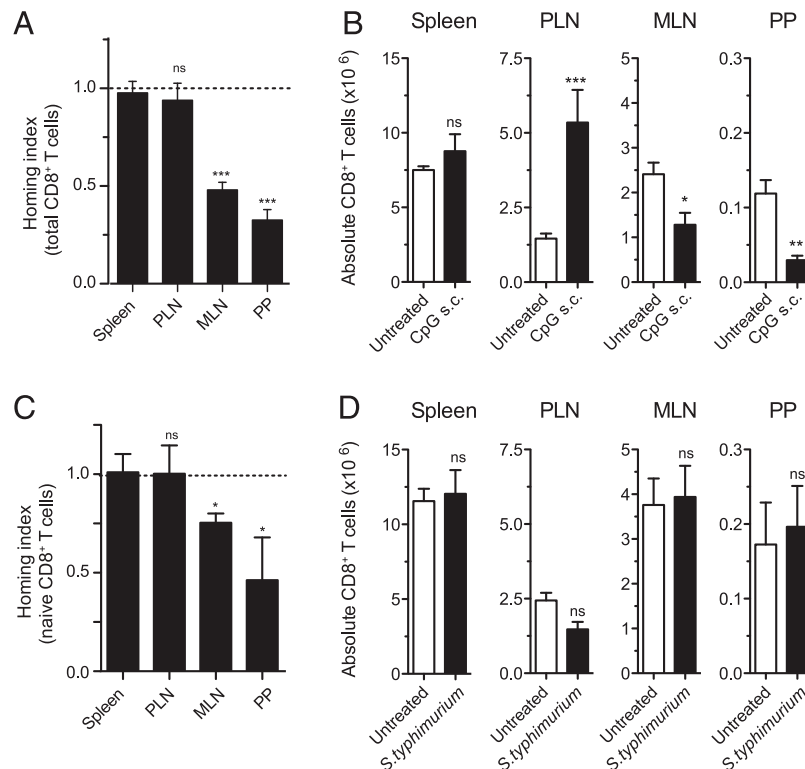
**FIGURE 4.**  $\alpha_4\beta_7$  downregulation is mediated by DCs and is dependent on IL-6. (A) Expression of  $\alpha_4\beta_7$  was measured by flow cytometry on CD8<sup>+</sup> T cells after total splenocytes, purified splenic CD8<sup>+</sup> T cells or DC-depleted splenocytes (CD11c<sup>-</sup> splenocytes) were cultured in the presence of CpG. (B) Expression of  $\alpha_4\beta_7$  on purified splenic CD8<sup>+</sup> T cells cocultured with BMDCs separated by a transwell membrane (BMDC transwell) or the culture supernatant of previously CpG-activated DCs (BMDC supernatant). Data show the  $\alpha_4\beta_7$  mean fluorescence intensity (MFI) on CpG-activated CD8<sup>+</sup> T cells compared with the MFI on unstimulated T cells in percentages. (C) Expression of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells after total splenocytes were cultured in the presence of increasing concentrations of recombinant cytokines. The dashed line represents  $\alpha_4\beta_7$  expression of CpG-activated cells. (D) Expression of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells after total splenocytes from wild-type mice and mice deficient for the indicated cytokines were cultured in the presence of CpG. Data show the  $\alpha_4\beta_7$  MFI on activated CD8<sup>+</sup> T cells compared with the MFI on unstimulated T cells in percentages. The different wild-type controls represent age- and strain-matched controls for each cytokine-deficient strain. All results are representative of at least two independent experiments. All data give the mean of quadruplicates  $\pm$  SEM. An asterisk without brackets indicates comparison with unstimulated CD8<sup>+</sup> T cells. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

DCs are known to secrete a variety of cytokines upon TLR stimulation (31). Accordingly, CpG-activated splenocytes produced high amounts of IL-6, IL-10, and IL-12p70 whereas IL-4 and IFN- $\alpha$  were below detection level (Supplemental Fig. 3A) as described for the CpG sequence used (32). To investigate the potential role of these cytokines for  $\alpha_4\beta_7$  downregulation, we cultured splenocytes in the presence of recombinant cytokines. We observed that IL-6 induced downregulation of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells (Fig. 4C), whereas the other cytokines examined had no influence on  $\alpha_4\beta_7$  expression. Following CpG stimulation of IL-6-deficient splenocytes,  $\alpha_4\beta_7$  downregulation on CD8<sup>+</sup> T cells was less marked than on wild-type cells (Fig. 4D). CpG stimulation of splenocytes from mice that lack IL-10 or IL-12p70 resulted in  $\alpha_4\beta_7$  downregulation that was comparable to wild-type cells. The cytokines IL-1 $\beta$ , IL-2, IL-15, and TNF- $\alpha$  can be released at low levels by TLR-activated DCs (31), but CD8<sup>+</sup> T cells did not show downregulation of  $\alpha_4\beta_7$  when splenocytes were cultured in the presence of these cytokines (Supplemental Fig. 3B). These data collectively demonstrate that loss of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells following TLR activation in vitro is mediated by DCs and is dependent on IL-6.

#### CpG activation blocks trafficking of CD8<sup>+</sup> T cells to GALT

To determine whether the TLR-induced downregulation of  $\alpha_4\beta_7$  affects trafficking of naive CD8<sup>+</sup> T cells, we examined the in vivo migration pattern of CD8<sup>+</sup> T cells after activation with CpG.

Fluorescently labeled CpG-activated splenocytes were adoptively transferred into naive recipient mice and their distribution in secondary lymphoid organs was compared with that of cotransferred unstimulated cells. Migration of CD8<sup>+</sup> T cells to the spleen and peripheral lymph nodes, which is independent of  $\alpha_4\beta_7$ , was not affected by CpG activation (Fig. 5A). In striking contrast, trafficking of CpG-activated CD8<sup>+</sup> T cells to the mesenteric lymph nodes and to the Peyer's patches was strongly impaired in comparison with the unstimulated transferred cells, as demonstrated by a homing index of 0.35. We next investigated whether these changes in the trafficking pattern of CD8<sup>+</sup> T cells can translate into an altered T cell distribution in secondary lymphoid organs during a prolonged TLR-associated inflammatory state. Because we have demonstrated that  $\alpha_4\beta_7$  downregulation is a self-limiting process and that expression levels of the adhesion molecules had nearly returned to baseline levels 48 h after a single TLR stimulus, we chose to perform repetitive TLR treatments to mimic an ongoing inflammatory process as found during infection. Four CpG injections at 2-d intervals resulted in clearly reduced numbers of CD8<sup>+</sup> T cells in intestinal lymphoid tissues (Fig. 5B). In contrast, the number of CD8<sup>+</sup> T cells was strongly increased in peripheral lymph nodes. These data suggest that TLR activation selectively impairs the ability of CD8<sup>+</sup> T cells to enter intestinal lymphoid organs. CD8<sup>+</sup> T cells numbers in Peyer's patches returned to baseline level 5 d after the last treatment with CpG, in accordance



**FIGURE 5.** CpG activation blocks trafficking of CD8<sup>+</sup> T cells to GALT. **(A)** Splenocytes were stimulated with CpG, fluorescently labeled, and coinjected with an equal number of labeled unstimulated cells i.v. into naive recipient mice. Secondary lymphoid organs were analyzed for adoptively transferred CD8<sup>+</sup> T cells by flow cytometry. The homing index was calculated as the ratio of CpG-activated CD8<sup>+</sup> T cells to unstimulated CD8<sup>+</sup> T cells in the indicated tissue corrected for the input ratio of transferred cells. Data show the mean values of individual recipient mice ( $n = 10$ ) from five independent experiments  $\pm$  SEM. The asterisk indicates comparison with the homing index for the spleen. **(B)** Mice were treated four times at 2-d intervals with CpG s.c. The diagram shows absolute numbers of CD8<sup>+</sup> T cells in secondary lymphoid organs of CpG-treated mice ( $n = 4$ )  $\pm$  SEM. **(C)** Unstimulated, fluorescently labeled splenocytes were injected i.v. into naive recipient mice prior to oral infection with *S. typhimurium*. Two days after infection, secondary lymphoid organs were analyzed for adoptively transferred CD8<sup>+</sup> T cells by flow cytometry. The homing index was calculated as the ratio of transferred naive CD8<sup>+</sup> T cells in the indicated tissue in *Salmonella*-infected mice to the number of transferred naive CD8<sup>+</sup> T cells in the same tissue in uninfected mice. Data show the mean values of individual recipient mice ( $n = 4$ )  $\pm$  SEM. **(D)** Two days after infection, absolute numbers of endogenous CD8<sup>+</sup> T cells were analyzed in secondary lymphoid organs. Data give the mean value of CD8<sup>+</sup> T cell numbers from *Salmonella*-infected mice ( $n = 5$ )  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

with the transient nature of  $\alpha_4\beta_7$  downregulation (Supplemental Fig. 4A). During systemic *S. typhimurium* infection, naive CD8<sup>+</sup> T cells also showed reduced trafficking to intestinal lymphoid organs, whereas migration to spleen and peripheral lymph nodes was not altered 42 h after infection (Fig. 5C). However, in contrast to CpG stimulation, *S. typhimurium* infection did not lead to a decrease in the total CD8 T cell numbers in mesenteric lymph nodes or the Peyer's patches (Fig. 5D). In summary, we show in this study that TLR activation downregulates the expression of the gut-homing integrin  $\alpha_4\beta_7$  specifically on naive bystander-activated CD8<sup>+</sup> T cells and that trafficking of such TLR-activated T cells to GALT is largely blocked.

## Discussion

The trafficking of circulating naive T cells is assumed to be non-competitive and to follow a random distribution within the secondary lymphoid organs (33). Our observation that *S. typhimurium* infection critically alters the expression pattern of a tissue-specific homing molecule on naive CD8<sup>+</sup> T cells is therefore unexpected. The observed downregulation of  $\alpha_4\beta_7$  was not restricted to *Salmonella* infection but was also shown upon stimulation with *E. coli* or synthetic TLR agonists, demonstrating that  $\alpha_4\beta_7$  downregulation is a broad reaction pattern following innate immune activation. Importantly, this effect is reversible after the resolution of an inflammation, as  $\alpha_4\beta_7$  expression returned to baseline levels 48 h after a single TLR stimulus. We have demonstrated that the CD8<sup>+</sup> T cells that downregulate  $\alpha_4\beta_7$  are activated upon TLR stimulation in the absence of their cognate Ag. These CD8<sup>+</sup> T cells are characterized by the naive CD62L<sup>high</sup>CD44<sup>low</sup> phenotype, they do not proliferate, and they show high surface expression of the activation marker CD69, consistent with the previously described bystander-activated phenotype (11, 30). In contrast, CD8<sup>+</sup> T cells specific for an OVA epitope showed marked upregulation of  $\alpha_4\beta_7$  in the GALT associated with proliferation upon immunization with OVA and a TLR ligand, in accordance with previous reports (27). In the spleen, Ag-specific CD8<sup>+</sup> T cells expressed intermediate  $\alpha_4\beta_7$  levels as described (7) (data not shown). These findings demonstrate that TLR activation leads to opposite effects on  $\alpha_4\beta_7$  expression of CD8<sup>+</sup> T cells at different stages of differentiation. Whereas TLR costimulation boosts site-specific upregulation of the gut-homing molecules  $\alpha_4\beta_7$  and CCR9 on Ag-specifically-activated CD8<sup>+</sup> T cells (27), naive T cells show downregulation of  $\alpha_4\beta_7$  without an increase in CCR9 upon TLR stimulation.  $\alpha_4\beta_7$  expression was on average higher on CD8<sup>+</sup> T cells after in vitro culture than in freshly isolated cells, even in the absence of stimulation. However, even when different in absolute levels,  $\alpha_4\beta_7$  was decreased following CpG activation both in vivo and in vitro (for direct comparison, see Supplemental Fig. 4B).  $\alpha_4\beta_7$  downregulation was also observed on CD4<sup>+</sup> T cells following TLR stimulation, although the decrease differed greatly between CD4<sup>+</sup> T cell subsets (Supplemental Fig. 3C). This effect is currently under investigation in our laboratory.

In transfer experiments we have shown that TLR stimulation drastically impairs naive CD8<sup>+</sup> T cell migration to GALT. Recirculation to spleen or peripheral lymph nodes, alternatively, was not altered by the TLR stimulus. This homing pattern resembles the migratory capacity of T cells that either genetically lack the  $\beta_7$  subunit or have been treated with neutralizing Abs against the  $\alpha_4\beta_7$  heterodimer or its subunits (5, 34, 35). Lymphocytes functionally deficient in  $\beta_7$  show a radically impaired homing to the Peyer's patches but little change in their recirculation to mesenteric lymph nodes due to the redundant activity of the adhesion molecule CD62L, which can partly compensate for the absence of

$\alpha_4\beta_7$  (36). In our model of TLR activation, homing of bystander-activated CD8<sup>+</sup> T cells was impaired for both mesenteric lymph nodes and Peyer's patches, despite persistent expression of CD62L. Thus, the homing pattern of TLR-activated CD8<sup>+</sup> T cells is similar to that of  $\beta_7$ -deficient cells, suggesting that  $\alpha_4\beta_7$  downregulation controls reduced trafficking to GALT following innate immune activation. Even though  $\alpha_4\beta_7$  was similarly downregulated during *S. typhimurium* infection, the absolute number of naive CD8<sup>+</sup> T cells in intestinal lymphoid organs was not decreased, in contrast to the strong reduction in CD8<sup>+</sup> T cells following TLR stimulation with synthetic CpG. CD8<sup>+</sup> T cell counts are most likely influenced by simultaneously occurring phenomena during bacterial infection, such as the clonal expansion of Ag-specific-activated T cell populations.

Coculture experiments showed that DC-released humoral factors are necessary and sufficient to mediate CD8<sup>+</sup> T cell  $\alpha_4\beta_7$  downregulation upon CpG stimulation. DCs are known to stimulate effector functions in T cells in the absence of nominal Ag, both by forming stable conjugates with naive T cells (37, 38) and by secreting soluble factors (39). Upon stimulation with a panel of TLR ligands, bystander activation of naive T cells has been shown to be mediated by a combination of DC-released type I IFN and NK cell-produced IFN- $\gamma$  (11). In this study, we show that  $\alpha_4\beta_7$  downregulation in vitro is to a large extent dependent on IL-6, which is produced abundantly by splenocytes upon CpG stimulation. Similarly, the decrease in  $\alpha_4\beta_7$  following *S. typhimurium* infection in vitro was also dependent on IL-6 (data not shown). IL-6 has been described to promote Ag-independent activation of naive CD8<sup>+</sup> T cells in the absence of specific TCR stimulation (12). However,  $\alpha_4\beta_7$  downregulation was not entirely abrogated in IL-6-deficient cells, suggesting that upon TLR activation, DCs produce other soluble factors in addition to IL-6 that contribute to the downregulation of  $\alpha_4\beta_7$  on CD8<sup>+</sup> T cells.

A recent report has demonstrated that during *S. typhimurium* infection, B cells shed CD62L, resulting in decreased B cell numbers in the lymph nodes and increased trafficking to the spleen (40). The authors speculated that these changes in B cell migration may contribute to the regulation of the inflammatory response. Similarly, the  $\alpha_4\beta_7$  downregulation observed on bystander CD8<sup>+</sup> T cells upon innate immune activation may represent a regulatory mechanism to prevent the trafficking of these nonspecifically activated cells into intestinal lymphoid tissue. Indeed, nonspecific CD8<sup>+</sup> T cells that are activated in a bystander manner by TLR-associated cytokines show increased responsiveness to subsequent stimulation via their TCR (12). Additionally, such cytokine-primed CD8<sup>+</sup> T cells have been shown to react to self-Ags and thus to play a role in the pathogenesis of autoimmune diabetes in a mouse model (41). With the presence of food Ags and commensal bacteria, the gastrointestinal tract harbors an abundance of potential targets for unspecifically activated CD8<sup>+</sup> T cells, and cytokines that can trigger such bystander activation are augmented during microbial infection. Pre-armed bystander CD8<sup>+</sup> T cells may thus contribute to the pathogenesis of intestinal inflammatory disorders, and it may therefore be beneficial to transiently exclude them from the gastrointestinal tract through downregulation of  $\alpha_4\beta_7$ . The importance of  $\alpha_4\beta_7$  in perpetuation of chronic inflammatory disease is demonstrated by the clinical efficacy of mAbs that block the  $\alpha_4\beta_7$  heterodimer or its  $\alpha_4$  subunit in patients with autoimmune disorders (42, 43). Further studies are needed to confirm the physiological relevance of  $\alpha_4\beta_7$  downregulation in a model of intestinal inflammation. Generally, understanding the regulation of bystander-activated CD8<sup>+</sup> T cell trafficking in health and disease may open new possibilities for the treatment of immune pathologies.



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## Disclosures

The authors have no financial conflicts of interest.

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